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Decreased Fetal Weights in Rats Exposed to Sidestream Cigarette Smoke

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Pregnant Sprague–Dawley rats were exposed to sidestream cigarette smoke (SS) for 6 hr a day, at a concentration of 1 mg/m^3 of respirable total suspended particulate material (TSP) on Days 3, 6–10, and 13–17 of pregnancy. Controls were kept in an identical chamber without smoke exposure. The animals were killed on Day 20 of gestation. No differences were found in maternal body weight gain or average daily food consumption between the smoke-exposed and control groups. The numbers of fetuses and of implantation sites per litter were comparable among the groups. None of the pups showed any gross malformations and no difference was found between controls and SS-exposed pups when examined for reduced skeletal ossifications. However, there was a small but significant reduction in mean pup weight. We conclude that intermittent exposure of rats to sidestream cigarette smoke at concentrations severalfold greater than those encountered in smokey public indoor environments causes intrauterine growth retardation. © 1994 Society of Toxicology

It has been observed in women who actively smoke during pregnancy that the average weight of their babies is generally somewhat lower at birth, on average by about 200 g, than it is in babies born to nonsmoking mothers. Long-term studies on growth and development suggest that smoking during pregnancy may affect physical growth, mental development, and behavior of offspring (U.S. Department of Health, Education and Welfare, 1979). While this adverse health effect of smoking has been known for some time, there is less certainty whether exposure of pregnant women to environmental tobacco smoke (ETS) has a similar effect. In one study that examined the question it was estimated that the average birth weight of babies in mothers exposed to cigarette smoke from their smoking husbands was reduced by approximately 120 g for each pack of cigarettes smoked per day by the father (Rubin *et al.*, 1986). In another study it was found that exposure during pregnancy for at least 2 hr a day to ETS at home or at work reduced the average birth weight by 24 g (Martin and Bracken, 1986). However, the conclusions drawn from these studies have on occasion been questioned either because it appeared that confounding factors had not been taken into account or because observations made by other investigators did not confirm the original conclusions (Trichopoulos, 1986; MacArthur and Knox, 1987). Nevertheless, at least some evidence does suggest that exposure to ETS during pregnancy may produce a small degree of intrauterine growth retardation, similar to that seen in the offspring of active smokers.

Recently we have systematically explored effects of cigarette sidestream smoke (SS) on the lung development of rats exposed to a controlled atmosphere of SS while *in utero* and during the first 100 days of the postnatal period (Gebremichael *et al.*, 1993; Ji *et al.*, 1993). For the conduct of these experiments, we maintained a chamber concentration of 1 mg/m^3 of total respirable suspended particulate matter (TSP) produced by collecting reference cigarette SS. This target concentration was selected because it allows us to compare our observations with those of other studies. Some experiments suggest that, although a value of 1 mg/m^3 of TSP corresponds to "extreme" conditions when compared with field measurements, it represents in rats a no-observed-effect level (NOEL; Coggins *et al.*, 1992, 1993). While conducting our research it was noted that average weights of pups from several randomly selected litters from SS-exposed pregnant rats collected on Day 21 of pregnancy appeared to be lower than the average weights of individual pups delivered by mothers exposed to filtered air. We decided to examine whether exposure of rats to SS during pregnancy would adversely affect the average litter weight. In this paper we report that SS exposure produced signs of a small, but significant, intrauterine growth retardation.

MATERIALS AND METHODS

Animals. Timed-pregnant Sprague–Dawley rats, 7 to 8 weeks old, were purchased from Zivic Miller Laboratories (Zelienople, PA). For each individual experiment, 16 rats were received on Day 2 of pregnancy, a Thursday (Day 0 being defined as the day following mating on which a sperm plug was found). Upon receipt, the animals from each shipment were assigned at random to two groups: SS-exposed and control group. The animals were housed during the entire experiment in plastic shoebox cages on conventional bedding.

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Four cages containing two animals each were placed within a 0.44-m^3 Hinners-type stainless-steel and glass inhalation chamber and exposed to SS. The rats were exposed to SS on Days 3 (the Friday after receipt of the animals), 6–10 (Monday through Friday of the second week of pregnancy), and 13–17 (Monday through Friday of the third week) for 6 hr per day, from 10:00 to 16:00 hr. The animals were kept around the clock in the exposure chamber which was ventilated during nonexposure hours with HEPA-filtered room air. The animals had free access to laboratory chow and water. Four control cages were placed in a similar chamber that was ventilated with HEPA-filtered air and that was located adjacent to the smoke exposure chamber. Food consumption in the SS-exposed animals was measured daily by weighing the food pellets, and the amount of food consumed in a day by the SS-exposed group was allotted the next day to the control animals.

Smoke exposure system. Sidestream smoke was generated by burning Kentucky 1R4F reference cigarettes and by drawing the sidestream smoke continuously into the inhalation chamber. The exposure system has been described in detail (Teague *et al.*, 1994). Briefly, reference cigarettes, stored at 4°C until needed, were conditioned at a relative humidity of 60% for at least 48 hr before use. The cigarettes were smoked by applying 35-ml puffs of 2-sec duration once every minute, for a total of eight puffs, and the smoke was collected in an absolute filter. The cigarettes were housed in a dilution chimney which collects and transfers only sidestream smoke, given off the glowing cigarette tips between puffs. The SS was drawn into a conditioning chamber (volume, 0.44 m^3) and, by changing the air flow through the chamber, diluted to a concentration of 4 mg/m^3 of TSP. After approximately 2 min of aging, the smoke was again diluted to a final concentration of 1 mg/m^3 of TSP and drawn into the exposure chambers. No significant differences in TSP concentrations were found whether TSP were measured inside or outside of the plastic cages, with and without animal bedding.

Characterization of chamber atmosphere. Details on the analytical procedures that were used to characterize chamber atmospheres are given by Teague *et al.* (1994). Briefly, TSP was measured in the conditioning chamber and within the exposure chamber by filter samples, a piezobalance (TSI Instruments, St. Paul, MN), and a PDM-3 MiniRam forward shattering device (MIE, Inc., Billerica, MA). Particle size distribution was measured using a Royco 236 laser particle counter (HIAC/Royco Instruments, Menlo Park, CA). The count median diameter was 0.228 ± 0.12 μm (geometric SD 4.2 ± 1.4). Carbon monoxide was monitored in the conditioning chamber and the exposure chamber with a Model 880 nondispersive-infrared (NDIR) analyzer (Beckmann Industrial, La Habra, CA). Nicotine concentrations in the exposure chambers were measured by drawing air samples every 15 min through sorbent tubes. The tubes were extracted with HPLC-grade ethyl acetate modified with 0.1% triethylamine and the extract was analyzed in a gas chromatograph (Varian 3740) equipped with a DB-5 30-m × 0.53-mm column (film thickness 1.5 μm) and a nitrogen-selective thermionic-specific detector. Temperature and relative humidity within the chambers were monitored with an appropriate probe located within the chamber (Rustrack, St. Paul, MN).

Termination of animals. On Day 20 of gestation, all animals were euthanized by pentobarbital overdose. The number of live and dead fetuses and the number of resorptions/abortions were recorded for each animal. The fetuses were weighed individually. Approximately half of the fetuses were immersed in 70% ethanol (the other half were fixed in Bouin's solution), eviscerated, skinned, cleared in 1% KOH, and stained for skeletal examination with Alizarin Red S (Taylor, 1986).

Plasma nicotine and cotinine levels. Plasma nicotine and cotinine levels were determined in female virgin rats, 8 to 12 weeks old, exposed under conditions identical to those used for the pregnant rats. Concentrations of the two compounds were determined by gas chromatography with nitrogen–phosphorus detection (Jacob *et al.*, 1981). This method has been modified for simultaneous extraction of nicotine and cotinine and determination using capillary gas chromatography (Jacob *et al.*, 1991). A Hewlett-Packard Model 5890 (Hewlett-Packard, Avondale, PA) instrument was used with a DB-5 capillary megabore column, 30 m × 0.53 mm i.d. (J&W Scientific Co., Folsom, CA) with a nitrogen–phosphorus detector. A helium flow velocity of 18 cm/sec and nitrogen makeup gas flow of 19.9 ml/min were used; injection port and detector temperature were 250 and 300°C, respectively. The internal standards, 5-methylnicotine and 5-methylcotinine, were obtained from Jacob Peyton, III (Division of Clinical Pharmacology, Department of Medicine, University of California, San Francisco).

Statistical analysis. The data obtained in controls and in SS-exposed animals from different experiments were analyzed by Kruskal–Wallis nonparametric ANOVA. When it was determined that the replicate experiments could be pooled, all parameters (maternal body weights, implantation/resorption sites, number of live/dead fetuses per litter) were calculated as means and SE. For the evaluation of birth weights, an average value from all individual pup weights for each litter was calculated and this number was taken as average pup weight per litter. Comparisons between control and treated groups were done with Student's *t* test and a p value of 0.05 or less was considered to be significant.

RESULTS

Data on exposure conditions are presented in Table 1. Under these conditions, the average plasma nicotine level in virgin female rats was 11.5 ± 7.2 ng/ml (n = 24) measured following a 6-hr exposure. Plasma cotinine levels were 32.0 ± 22.0 ng/ml (n = 5) following 1 day of exposure and then accumulated, until after the last exposure on Day 5, when they reached 98.0 ± 10.0 ng/ml of plasma. The plasma cotinine levels are comparable to those found by Coggins *et al.* (1992) in Sprague–Dawley rats exposed to 1 mg/m^3 of particulates. The results are also consistent with known clearance rates of nicotine and cotinine in Sprague–Dawley rats (Plowchalk *et al.*, 1992).

In the experiments designed to examine the effects of SS on fetal and postnatal lung development (Ji *et al.*, 1993; Gebremichael *et al.*, 1993), selected offspring from different litters were collected on Day 21 of pregnancy and after birth. It was noted in these experiments that average weights of pups from several randomly selected litters on Day 21 of pregnancy were 5.8 ± 0.1 g (SE; n = 12) in smoke-

TABLE 1
Average Exposure Data[a]

Parameter	Average	SD	n
Relative humidity (% RH)	28.9	10.4	16
Temperature (°C)	23.2	3.4	16
Carbon monoxide (ppm)	5.5	0.7	16
Nicotine ($\mu g/m^3$)	405	149	16
Total suspended particulate matter, TSP Mg/m^3	1.0	0.1	16

[a] Chamber concentrations of carbon monoxide, nicotine, and TSP were measured daily while pregnant rats were exposed to SS and the data shown represent typical average results obtained during the exposures.

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exposed rats as compared to 6.5 ± 0.3 g (SE; n = 14) in the offspring of unexposed control animals. At birth, the average weight of neonates born to mothers exposed to SS during pregnancy was 7.5 ± 0.1 g (SE; n = 8). The average weight of pups born to sham-exposed mothers was 9.1 ± 0.3 g (SE; n = 8). All these numbers represent means from individual animals. To confirm these observations, it was decided to examine the effects of SS exposure on average pup weight per litter.

Totals of 16 pregnant controls and 15 pregnant and SS-exposed animals were available and were obtained in two different and independent experiments. The data on maternal weight gain during pregnancy and on litter characteristics from the two experiments were pooled and are presented in Table 2. It can be seen that the average weight gain of smoke-exposed rats during pregnancy was practically identical to that of controls. There was no difference in number of implantation sites, number of live or dead pups per litter, or number of animals with resorptions. The only difference between the two groups was a small but significantly lower than average pup weight per litter in the smoke-exposed animals.

None of the pups showed any gross malformations upon inspection. Twenty-four pups from the control groups and 51 pups from the smoke-exposed groups were selected at random and examined for skeletal ossification. The data are presented in Table 3. Normal ossification was observed in four of the six skeletal zones examined (sternebrae, lumbar vertebrae, metacarpals, and metatarsals). In two other zones (thoracic and caudal vertebrae), there was a slight, statistically insignificant increase in the incidence of reduced skeletal ossifications. No skeletal variations or malformations were noted in either group.

TABLE 2
Maternal Body Weights and Litter Weights in Rats Exposed to Sidestream Smoke[a]

	Smoke-exposed[b]	Controls[b]
Maternal body weight, Day 2 (g)	240 ± 6	239 ± 4
Maternal body weight, Day 20 (g)	397 ± 11	392 ± 8
Implantation sites per litter	14.0 ± 0.7	12.6 ± 0.7
Resorptions/abortions per litter	1.2 ± 0.5	1.1 ± 0.7
No. of animals with resorptions	5/15	4/16
No. of live pups per litter	12.5 ± 1.0	11.3 ± 0.8
No. of dead pups per litter	0.3 ± 0.1	0.3 ± 0.1
Average pup weight per litter (g)	4.05 ± 0.07*	4.36 ± 0.10

[a] Pregnant rats were exposed to sidestream smoke as indicated under Materials and Methods and were killed on Day 20 of pregnancy. All parameters listed are average values per litter from two separate experiments.

[b] Values are given as mean and SE; number of litters for smoke exposed animals n = 15, for controls n = 16.

* $p < 0.05$ compared to controls.

TABLE 3
Rat Fetus Skeletal Analysis

		Smoke-exposed	Controls
Sternebrae	Normal	51/51 (100%)	24/24 (100%)
	Reduced	—	—
Thoracic	Normal	42/51 (82%)	22/24 (92%)
	Reduced	9/51 (18%)	2/24 (8%)
Lumbar	Normal	51/51 (100%)	24/24 (100%)
	Reduced	—	—
Caudal	Normal	50/51 (98%)	24/24 (100%)
	Reduced	1/51 (2%)	—
Metacarpals	Normal	51/51 (100%)	24/24 (100%)
	Reduced	—	—
Metatarsals	Normal	51/51 (100%)	24/24 (100%)
	Reduced	—	—

Note. The incidence of reduced ossification sites was not significantly different between smoke-exposed animals and controls as determined by χ^2 analysis.

DISCUSSION

We exposed pregnant rats to SS generated from burning Kentucky 1RF4 reference cigarettes. In order to minimize stress and its potential adverse effects on gestation, a whole-body exposure system was used rather than keeping the rats in nose-only restraint tubes during smoke exposure. From our experiments we conclude that exposure of pregnant rats to SS at TSP of 1 mg/m^3 affects fetal development. The reduction in birth weight was comparatively small (7%) and not accompanied by a significant decrease in ossification. This suggests that exposure to SS under the present conditions produced small but significant intrauterine growth retardation. Decreased overall fetal weight in rats exposed to mainstream cigarette smoke has been described previously in several studies (Essenberg *et al.*, 1940; Younoszai *et al.*, 1969; Tachi and Aoyama, 1983; Leichter, 1989). The extent in fetal weight reduction ranged from being comparable to what was found in our experiments (approximately 5% weight reduction; Peterson *et al.*, 1981) to a fetal weight reduction of 70% of control values in one study using extreme exposure conditions (Reznik and Marquard, 1980). Unfortunately, no direct comparison between the different experiments is possible because of the different exposure regimens (days of exposure, length of exposure to mainstream smoke) and because no analytical data on chamber atmospheres are available. At best, exposure conditions were characterized by indicating the number of cigarettes burned. This precludes direct comparisons of our findings on fetal development with other data in the literature.

The chamber atmosphere in our experiments was in all likelihood less concentrated than in most previous experiments in which pregnant rats were exposed to cigarette smoke. A concentration of 1 mg/m^3 of TSP is roughly 10

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times higher than what can be measured on average when there is indoor smoking, where concentrations as high as 300 $\mu g/m^3$ occur only rarely (Guerin *et al.*, 1992). Likewise, common public exposure to nicotine is on the average 10 $\mu g/m^3$, although under higher exposure conditions it may increase up to 10 times this value. On the other hand, exposure to CO in our experiments is comparable to the average public exposure of 4–5 ppm in restaurants and 2–3 ppm in the workplace (Guerin *et al.*, 1992). It should be pointed out in this context that exposure of rats for up to 90 days under conditions similar to ours failed to produce any signs of histopathological lesions in the respiratory tract (Coggins *et al.*, 1992, 1993). In those experiments, a concentration of 1 mg/m^3 of SS-generated particulate matter in the ambient air was considered to represent a no-observable-effect level. At higher concentrations, such as 4–10 mg/m^3 of TSP, histopathological changes seen in the respiratory tract were considered to be minimal and potentially reversible (Coggins *et al.*, 1993; Von Meyerinck *et al.*, 1989). From our observations we must conclude that fetal weight may represent a more sensitive indicator of exposure to SS.

What mechanisms might account for our observation of intrauterine growth retardation in fetuses of rats whose dams were exposed to SS? The precise underlying mechanisms of the harmful fetal effects related to maternal smoking (exposure of the fetus to mainstream cigarette smoke or its products) during pregnancy are controversial. Suggested mechanisms of action, which could also explain the effects observed in this study with SS, include nicotine-induced vasoconstriction, resulting in a chronic reduction in placental blood flow, and fetal hypoxia due to CO in smoke (Tachi and Aoyama, 1983, 1986). The underlying pathogenic mechanism for nicotine described by most investigators is a constriction of the uteroplacental arteries, mediated through the release of epinephrine by the nicotine, causing reduced inflow of maternal blood into the placenta and reduced placental nutrient transfer (Resnik *et al.*, 1979; Philipp *et al.*, 1984; Nash and Persaud, 1988). Since the vasoactive effect of nicotine is mediated by the release of epinephrine, one would expect that the effects of nicotine and epinephrine on fetal development would be similar. However, data published by Trend and Bruce (1989) indicate that a sevenfold elevation of plasma epinephrine levels in pregnant rats does not affect fetal development, suggesting that vasoconstriction induced by epinephrine alone does not cause fetal growth retardation. Thus, some other nicotine-mediated mechanisms or other bioactive compounds of tobacco smoke may be responsible for the changes in fetal development. Unilateral ligation of the uterine artery in the early stages of gestation in the rat, a more drastic intervention in uterine blood flow, also has little effect on fetal development (Bar and Brent, 1970; Bruce, 1976). Thus the question of whether ETS-induced inhibition of intrauterine fetal development is indeed caused by nicotine-mediated vasoconstriction or whether CO or other chemicals in ETS play a contributory role is certainly an unresolved issue.

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